Applied and Environmental Microbiology, Mar. 2009, p. 1525–1533 Vol. 75, No. 6
0099-2240/09/$08.00+0 doi:10.1128/AEM.02261-08

Bacteroidales Diversity in Ring-Billed Gulls (*Laurus delawarensis*) Residing at Lake Michigan Beaches[▽]

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Received 1 October 2008/Accepted 8 January 2009

This study investigated the occurrence and diversity of *Bacteroidales* fecal bacteria in gulls residing in the Great Lakes region. Members of this bacterial order have been widely employed as human and bovine host-specific markers of fecal pollution; however, few studies have focused on gulls, which can be a major source of fecal indicator bacteria and pathogens at beaches. We found a low but consistent occurrence of *Bacteroidales* in gulls at five beaches in three different counties spanning the Wisconsin shoreline of Lake Michigan. The percentages of gulls positive for *Bacteroidales* were 4 to 8% at beaches in the southern part of the state and 8 to 50% at beaches in the north. Sequencing of 931 clones from seven gull *Bacteroidales* 16S rRNA gene libraries revealed a large amount of diversity in both individual and pooled gull fecal samples. Two libraries constructed from pooled gull fecal samples ($n = 5$ and $n = 6$) did not have a greater richness of sequences than individual samples, suggesting that even within a single gull diversity is high and an extensive sequencing effort is needed to characterize the populations. Estimates of the numbers of operational taxonomic units (OTUs) for the libraries obtained using different similarity levels revealed a large amount of microdiveristy with a limited number of OTUs at the 95% similarity level. Gull sequences were clustered by the beach from which they were collected, suggesting that there were geographic effects on the distribution of *Bacteriodales*. More than 53% of the 16S rRNA gene sequences from gulls at the southern beaches were associated with the family *Porphyromonadaceae*, primarily the genus *Parabacteroides*, whereas sequences from gulls at the northern beaches were comprised of *Bacteroidaceae* and *Prevotellaceae* sequences. Comparison of gull sequences with sequences from goose, canine, raccoon, and sewage sources revealed distinct clusters of closely related gull sequences; however, these sequences were widely dispersed across a dendrogram that included all other sources, including previously characterized gull *Bacteroidales* from other studies, suggesting that geographic influence or simply sample representation plays a greater role in the observed population structure than strictly the host gut environment.

Problems with recreational water quality have been widely reported for Great Lakes beaches (13, 24, 28, 35, 36). *Escherichia coli* has been the most commonly used indicator of fecal contamination in the Great Lakes region. This bacterium is commonly found in the gastrointestinal tracts of warm- and cold-blooded animals. For Great Lakes beaches, nearly 90% of the water quality advisories have been reported to be due to unknown causes (C. Kovatch, presented at the U.S. EPA National Beach Conference, Niagara Falls, NY, 11 to 13 October 2006), which highlights the need for better indicators for detecting and identifying sources of fecal pollution so that remediation efforts can be implemented.

Beaches have been found to be contaminated by a variety of sources, including sewage discharges, urban storm water, and roosting waterfowl and gulls (11, 33). One study demonstrated that there was an approximately 500-fold increase in fecal indicator bacteria when gulls were attracted to a beach area (21). Other studies have shown that gull feces contain a large and variable population containing both *E. coli* and enterococci (10^5 to 10^9 CFU g^{-1} and 10^4 to 10^8 CFU g^{-1}, respectively) and could contribute these indicator organisms to beach water in the Great Lakes (11). Gull feces also have been shown to contain a variety of fecal bacteria pathogenic to humans, such as *Salmonella*, *Campylobacter*, *Aeromonas*, and *Yersinia* (16, 20, 21, 27).

Certain *Bacteroides* and *Prevotella* spp. have been employed as host-specific markers of fecal pollution. Since culture techniques for isolation of these anaerobic bacteria are difficult to perform, molecular techniques have been developed to amplify, detect, and in some cases quantify the 16S rRNA genes of *Bacteroides* spp. from feces and water (4, 9, 15, 17, 19, 23). Previous research has focused primarily on identifying *Bacteroidales* found in human and bovine sources, and some studies have also included swine and equine sources (3, 4, 6, 12, 15, 18, 19, 23, 25). Characterizations of *Bacteroidales* in other host sources of fecal pollution have included dogs, cats, elk, geese, horses, pigs, and seagulls (9, 12, 15). The utility of the markers has been tested extensively in contaminated environments, including beaches (1, 5, 29). While these studies offer some insight into the population structure of *Bacteroidales*, limited numbers of animals in defined geographical areas have been characterized, preventing a thorough assessment of the potential usefulness and limitations of employing members of *Bacteriodales* as host-specific markers of fecal pollution.

Identification of host sources provides information concern-

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▽ Published ahead of print on 16 January 2009.

TABLE 1. Percentages of gull fecal samples collected at Lake Michigan beaches that were positive for *Bacteroidales* spp. as determined by PCR

Beach	County	Location	% Advisories/ % closings	Sampling time	No. of gulls sampled	% Positive for *Bacteroidales*
Bailey's Harbor	Door	Northern	1/0	May to September 2005	25	8.0
Whitefish Dunes	Door	Northern	1/3	May to September 2005	15	26.7
Murphy Park	Door	Northern	5/0	May to September 2006	8	12.5
Menominee Park	Winnebago	Northern	NM[a]	2006	4	50
Bradford	Milwaukee	Southern	56/4	May to September 2004	225	4.8
North Beach	Racine	Southern	20/3	May to September 2004	190	8.4

[a] NM, not monitored (inland lake).

ing the likelihood of the concurrent presence of certain pathogens and may prove to be a useful surrogate for testing directly for pathogens. For example, the presence of a human-specific *Bacteroides* genetic marker in beach water has been shown to correlate well with the occurrence of human enteric pathogens (30). Given the mandate of the Federal Beach Protection Act of 2008 (which amends the Beaches Environmental Assessment and Coastal Health Act of 2000) to develop and implement coastal recreational water pollution source identification and tracking programs for beaches (http://www.govtrack.us/congress/bill.xpd?bill=h110-2537), the importance of exploring alternative taxa for these purposes has increased greatly.

We investigated the distribution and population structure of *Bacteroidales* in gulls at beaches along western Lake Michigan. Some beaches had ongoing microbial contamination problems, and some were within the Environmental Protection Agency's recommended limits (34) for most of the beach season. In addition, we compared the diversity and lineage of *Bacteroidales* in gull feces with the diversity and lineage of *Bacteroidales* in fecal waste from other animals and from sewage. It appears that some gulls are colonized by members of *Bacteroidales*, and there is a high level of similarity of these members in gulls at beaches close to each other. Further, members of the *Bacteroidales* from gulls appear to be more similar to each other than to members of the *Bacteroidales* from other animals.

MATERIALS AND METHODS

Study area. From 2004 through 2006, gull fecal samples were collected at multiple beaches spanning the western shore of Lake Michigan, as well as Lake Winnebago, an inland lake in east central Wisconsin (Table 1). The beaches included Whitefish Dunes State Park (44°55′23″N, 87°11′46″W), Bailey's Harbor Ridges Park (45°04′47″N, 87°05′43″W), and Murphy Park (45°01′37″N, 87°33′02″W) in Door County (northern Lake Michigan), Bradford Beach (43°03′42″N, 87°52′24″W) in Milwaukee County, and North Beach (42°44′27″N, 87°46′45″W) in Racine County (southern Lake Michigan). Samples were also collected at Menominee Park (Lake Winnebago) (44°02′76″N, 88°52′01″W) in Winnebago County, WI. This inland lake in northeastern Wisconsin is approximately 30 miles west of Lake Michigan and was sampled to compare Lake Michigan gull populations with a neighboring gull population.

For comparison purposes, fecal samples were obtained from other sources, including three raccoon samples from two different locations in Milwaukee County, 54 goose samples from beaches at Otumba Park (44°50′03″N, 87°23′46″W), Murphy Park, Ellison Bay (45°15′20″N, 87°07′01″W), Egg Harbor (45°02′43″N, 87°18′04″W), and Sunset Park (44°52′19″N, 87°23′09″W) in Door County, and eight canine samples from Outagamie and Winnebago Counties in Wisconsin. Wastewater treatment plant (WWTP) influent samples were also tested and consisted of 24-h flow-weighted samples provided by the Milwaukee Metropolitan Sewage District in 2005 (3 samples), 2006 (2 samples), and 2007 (10 samples). The WWTP used in this study receives sewage from an approximately 500-km^2 service area.

Collection and processing of fecal and sewage samples. Fecal samples were collected from beach sand or from grass lawns surrounding the beach. A moist, mucoid sample was considered fresh, and a sample which appeared to be dry was considered not fresh. All fecal samples were collected in sterile Whirlpak bags or in sterile 15-ml centrifuge tubes using sterile tablespoons and then placed on ice and returned to the laboratory within 4 to 6 h. Fecal samples were stored at 4°C until bacterial DNA was extracted (no longer than 18 h). For WWTP samples, 100 ml of sewage influent was filtered onto a 0.45-μm nitrocellulose filter (Millipore, Bedford, MA), and the filters were stored in microcentifuge tubes at −80°C until DNA was extracted.

Extraction of bacterial DNA and PCR. Bacterial DNA was extracted from fecal samples using a QIAmp DNA stool mini kit according to the manufacturer's instructions (Qiagen Company, Valencia, CA). Approximately 1 g of gull feces was used for each extraction. Filtered sewage influent was extracted using an MPBIO FastDNA spin kit for soil (MP Biomedicals, Santa Anna, CA) according to the manufacturer's instructions, except that the cells were mechanically lysed using a MiniBeadBeater-8 cell disruptor (BioSpec Products, Bartlesville, OK) at the homogenization setting for 1.5 min at room temperature. DNA was stored in microcentifuge tubes at −20°C until it was analyzed. DNA concentration was determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE).

The presence of fecal bacterium DNA was confirmed by PCR analysis using primers uidA1318F and uidA1698R (5) that target the *uidA* gene of *E. coli*. Total *Bacteroidales* were detected using previously described primers Bac32F and Bac708R, and human-specific *Bacteroides* spp. were detected using primers HF183F and Bac708R (4). All reactions were performed using a *Taq* PCR Master Mix kit (Qiagen Co., Valencia, CA) with 7.5 pmol forward and reverse primers and between 10 and 80 ng of DNA per 25-μl reaction mixture. The thermocycler conditions used for PCR were as follows: one cycle of 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, the annealing temperature (60°C for the *uidA* primers, 53°C for the total *Bacteroidales* primers, and 59°C for the *Bacteroides* human-specific primers) for 30 s, and 72°C for 1 min, a final cycle of 72°C for 6 min, and then a hold at 10°C. PCR products were visualized on a 2% agarose gel stained with ethidium bromide and compared to a 100-bp DNA ladder molecular weight marker (Fisher Scientific Co., Pittsburgh, PA).

***Bacteriodales* clone libraries.** DNA templates used for PCR were obtained from DNA extracted from individual fecal samples, except for two gull libraries, for which multiple DNA extracts were pooled prior to cloning. These libraries included sequences for five gull fecal samples that were positive for total *Bacteriodales* from Bradford Beach in Milwaukee (designated Bradford pooled) and for six positive samples from North Beach in Racine (designated Racine pooled). Two of the three raccoon samples were also pooled prior to PCR and cloning (designated Rac-WA). All of the extracted DNA samples were normalized to obtain equal concentrations prior to pooling. For construction of the sewage library, DNA was extracted from a 24-h flow-weighted sample obtained from Jones Island WWTP in Milwaukee, WI.

PCR products were purified using a QIAquick PCR purification kit or a QIAquick gel extraction kit (Qiagen Co., Valencia, CA). PCR products were cloned using a Topo TA cloning kit with either One Shot Mach 1 or One Shot Top10 *E. coli* competent cells (Invitrogen Life Technologies, Carlsbad, CA). Transformants were transferred to microtiter plates containing 100 μl of Luria broth (LB) supplemented with 100 mg ml^{-1} ampicillin. An inoculated microtiter plate was covered with foil tape and then incubated overnight at 35°C for 18 h. After incubation, cells were centrifuged at 700 × *g*, and the medium was replaced with a 50:50 mixture of LB with 100 mg ml^{-1} ampicillin and 50% glycerol for long-term storage at −80°C.

Plasmid DNA was isolated using a manual method adapted to a 96-well format (M. Rise, personal communication). Briefly, cells were grown in 1 ml LB with 100 mg ml^{-1} ampicillin overnight. Cells were lysed with a 0.2 N sodium hydroxide–1% sodium dodecyl sulfate solution. The cell lysates were transferred to an AcroPrep 96-well filter plate (3.0-μm GF/0.2-μm BioInert; Pall Life Sciences, Ann Arbor, MI), which was used to remove cellular debris. The purified plasmid DNA was precipitated with 80% isopropanol and then resuspended in Tris-EDTA buffer (5 mM Tris-HCl, 0.05 mM EDTA; pH 8.5). Sequencing reactions were carried out with an ABI BigDye Terminator kit (Applied Biosystems, Foster City, CA). Clones were sequenced using either the M13 reverse primer (from the site on the vector) or the Bac32F primer. Sequencing was carried out using a BigDye Terminator v3.1 cycle sequencing kit according to the manufacturer's instructions, and sequencing reactions were run on an ABI Prism 3730 (Applied Biosystems, Foster City, CA). Single sequence reads were trimmed for quality using PHRED (10), which provided approximately 700-bp reads for further analysis.

Sequence analysis and bioinformatics. Sequences less than 400 bp long were removed from the data set. Sequences from each sample were aligned using ClustalW (32). CHECK_CHIMERA (8) was used to identify possible chimera sequences; between <1 and 3% of sequences from each library were removed. Sequence identity was verified by using NCBI BLAST (2) and the RPD II classifier (37). Each clone library was analyzed using DOTUR to calculate various estimates of bacterial diversity, including assignment to operational taxonomic units (OTUs) using the furthest-neighbor method (31). For construction of dendrograms, sequences were grouped using the CAP3 sequence assembly program (14) with an identity cutoff value of 97%, except for the *Porphyromonadaceae* dendrogram, where an identity cutoff value of 98% was used. Sequences with greater than 97% (or 98% for *Porphyromonadaceae*) identity were compiled into single consensus sequences for ClustalW alignments. For the gull sequences, neighbor-joining trees were constructed using 1,000 iterations to generate bootstrap values. The dendrogram was viewed in NJ Plot software (26). To construct a dendrogram of *Bacteroides* spp. from all sources (gull, goose, canine, raccoon, and sewage), 16S rRNA gene sequences belonging to this genus were identified using the RDP classifier. Sequences with 97% or greater identity to *Bacteroides* spp. were aligned using ClustalW, and a neighbor-joining tree was constructed using Bionumerics v5.1 software (Applied Maths, Kortrijk, Belgium). Similarly, clone libraries were screened using the RDP classifier for sequences with 95% or greater identity as members of *Porphyromonadaceae*, sequences were aligned with ClustalW, and neighbor-joining trees were constructed. Sequence data were managed using Vector NTI Advance sequence analysis software (Invitrogen Corporation, Carlsbad, CA). Identification of the HF183F primer sequence in each clone library was carried out using BLAST (2).

Nucleotide sequence accession numbers. All of the sequences determined in this study have been deposited in the GenBank database under accession numbers FJ219622 to FJ221366.

RESULTS

Occurrence of *Bacteroidales* in gulls. A total of 467 gull fecal samples from five coastal beaches spanning Lake Michigan's western shore and one inland beach on Lake Winnebago were screened for the presence of *Bacteroidales* by PCR. There was a low but consistent occurrence of *Bacteroidales* in the gull populations at these beaches (Table 1). A higher percentage of gulls were positive for total *Bacteroidales* at Whitefish Dunes Beach located along northern Lake Michigan than at the other beaches. In general, the occurrence of *Bacteroidales* in gulls at northern Lake Michigan beaches was higher than the occurrence of *Bacteroidales* in gulls at the two beaches in southern Wisconsin. For example, at Bradford and North Beaches only 4.8 and 8.4% of the samples, respectively, were positive.

Diversity of *Bacteroidales* within a single gull and pooled samples. *Bacteroidales* clone libraries were created with five individual gull fecal samples and two pooled samples from multiple gulls from Bradford Beach ($n = 5$) and North Beach ($n = 6$). The clone libraries were comprised of members of *Bacteroidaceae*, *Prevotellaceae*, and *Porphyromonadaceae*, primarily members of the genera *Bacteroides*, *Prevotella*, and *Parabacteroides*, respectively (Table 2). Members of these families were not uniformly distributed in the clone libraries from different gulls. Gulls from northern Lake Michigan beaches were found to contain primarily *Bacteroides* and *Prevotella* spp., whereas the majority of sequences recovered from gulls residing at southern Lake Michigan beaches were found to be sequences of members of the genera *Parabacteroides* and *Bacteroides*.

TABLE 2. Percentages of libraries classified as members of different taxa

Host	% of library classified as:			
	Bacteroidaceae	*Prevotellaceae*	*Porphyromonadaceae*	Unclassified[a]
Gull (northern beaches)	66	17		17
Gull (southern beaches)	45		53	1
Goose	80	14		6
Canine	95	5		
Raccoon	86	<1	13	<1
Sewage	76	11	2	

[a] Sequences were considered unclassified when there was less than 95% identity at the family level using the RDP II classifier.

All of the gull libraries were found to have similar levels of richness of sequences (Table 3), and the majority of sequences were found to be unique, suggesting that the *Bacteroidales* community in a single gull is complex rather than highly clonal. There were low numbers of OTUs at a 95% similarity cutoff for all libraries, suggesting that despite a large amount of microdiversity (e.g., >98% similarity), gulls harbor a limited number of subpopulations of *Bacteroidales*. The one exception was gull 85, in whose library there was nearly twice as many OTUs at the 99%, 97%, and 95% similarity cutoffs as in other gull libraries. Interestingly, this sample was obtained from the beach at which the occurrence of gulls positive as determined by PCR using primers directed against *Bacteroidales* was relatively high.

The clone libraries constructed from pooled gull fecal samples contained higher numbers of OTUs at levels of similarity of 99% and 97% than the individual samples, which was a function of the greater number of clones that were analyzed. This demonstrates that further sampling would yield additional OTUs and is an important consideration for the design of future studies to characterize *Bacteriodales* populations in hosts. At the 95% similarity level for an OTU, however, the clone libraries from pooled samples contained approximately the same numbers of OTUs as the libraries constructed from individual samples, despite the fact that almost 10-fold more clones were sequenced. Rarefaction analysis (described below for all hosts) revealed similar sampling saturation for individual versus pooled samples for all OTU criteria (unique and 99, 97, and 95% similarity), suggesting that even for individual animals greater diversity would be recovered with greater sampling.

Neighbor-joining trees were constructed from consensus sequences (from each library) of OTUs at the 97% similarity level. The majority of OTUs from each gull clustered more closely with each other than with the OTUs obtained from other gulls (Fig. 1). *Bacteroides* sequences from gull 85 (northern Lake Michigan) formed a distinct cluster containing 33 of

TABLE 3. Clone libraries used in this study and numbers of OTUs at levels of identity of 99%, 97%, and 95%

Clone library	Geographic location (beach/county)	Source	No. of clones sequenced	No. of unique sequences	No. of OTUs at 99% identity level	No. of OTUs at 97% identity level[a]	No. of OTUs at 95% identity level
Gull 50	Northern Lake Michigan, Bailey's Harbor/Door	Individual gull	22	21	12	7	5
Gull 85	Northern Lake Michigan, Whitefish Dunes/Door	Individual gull	41	41	30	20	12
Gull 134	Northeastern Wisconsin, Menominee Park/Winnebago	Individual gull	25	23	13	6	5
Gull 242	Southern Lake Michigan Bradford/Milwaukee	Individual gull	42	40	12	6	5
Gull 287	Southern Lake Michigan North/Racine	Individual gull	143	93	17	4	2
Pooled gull Bradford	Southern Lake Michigan, Bradford/Milwaukee	Five pooled samples	325	252	29	11	7
Pooled gull Racine	Southern Lake Michigan, North Beach/Racine	Six pooled samples	333	227	30	10	5
Goose 87	Northern Lake Michigan, Sunset Park/Door	Individual goose	12	12	9	8	6
Goose 117	Northern Lake Michigan, Murphy Park/Door	Individual goose	59	42	14	9	5
Goose 136	Northeastern Wisconsin, Menominee Park/Winnebago	Individual goose	40	36	27	12	10
Canine 133	Northeastern Wisconsin private residence/Outagamie	Individual dog	76	73	31	16	11
Raccoon 2	Southern Lake Michigan, Milwaukee	Individual raccoon	236	213	90	45	25
Raccoon WA	Southern Lake Michigan, Milwaukee	Two samples	76	74	19	9	7
Sewage influent	Southern Lake Michigan, Milwaukee	24-h flow-weighted sample	317	311	132	74	50

[a] Consensus sequences from individual samples that were more than 97% identical were used to construct overview neighbor-joining trees.

the 41 sequences in this clade. In addition, the other northern Lake Michigan sequences and the inland lake (northeastern Wisconsin) sequences appeared to be more closely related to each other than to sequences from gulls at the two southern locations. The libraries for the southern locations consisted of sequences from two individual gulls and two pooled samples from beaches located 45 km apart. The sequences recovered from these samples were intermixed in three distinct clades, and the majority of closely related North Beach (Racine, southern Lake Michigan) sequences in two adjoining clades and the Bradford Beach (Milwaukee, southern Lake Michigan) sequences were distributed among all three clades. One of these clades was identified as *Porphyromonadaceae*, with two branches showing intermediate (53 to 86%) and high (>93%) identities to *Parabacteroides* spp. (Fig. 1). Representative sequences of *Bacteroides*, *Prevotella*, and *Parabacteroides* spp. from other studies were included in the dendrogram for comparison. These previously characterized sequences were sequences from gulls and other hosts (human, cow, dog, and chicken) and did not fall close to the gull sequences obtained in this study, suggesting that the members of *Bacteriodales* in gulls from Lake Michigan are not well represented in these previously characterized data sets for other animals.

Comparison of *Bacteriodales* diversity and lineages in gulls with *Bacteriodales* diversity and lineages in other animals and sewage. A total of 54 goose samples were tested for the presence of total *Bacteroidales*, and 16.6% of these samples were positive. This frequency is similar to the frequency found in gulls. The three raccoon and eight canine samples were all positive for *Bacteroidales*. All 15 sewage samples from WWTP influent were also positive. The 16S rRNA gene sequences from these different sources were compared to the sequences found in gulls. Canine, raccoon, and sewage samples all were found to have a greater richness of unique sequences, as well as greater richness of OTUs at levels of identity of 99%, 97% and 95% (Table 3). Similar to the results for gulls, there was a large amount of microdiversity, and there were a limited number of OTUs at the 95% identity level; however, sewage samples and one raccoon sample contained considerably more OTUs than avian samples. The goose samples had a level of sequence richness similar to that of the gull samples. Rarefaction analysis of clone libraries showed that there were similar amounts of sample saturation in avian sources, including pooled and individual samples (Fig. 2), which suggests that there is relatively low diversity in this source. The only exception was gull 85. In contrast, canine samples, one raccoon sample, and the sewage sample had greater diversity than the avian samples.

To examine the relationship among *Bacteroides* spp. recovered from gull and other samples, sequences identified as *Bacteroides* spp. with 97% or greater identity using the RDP classifier were included in a phylogenetic tree. The percentage of

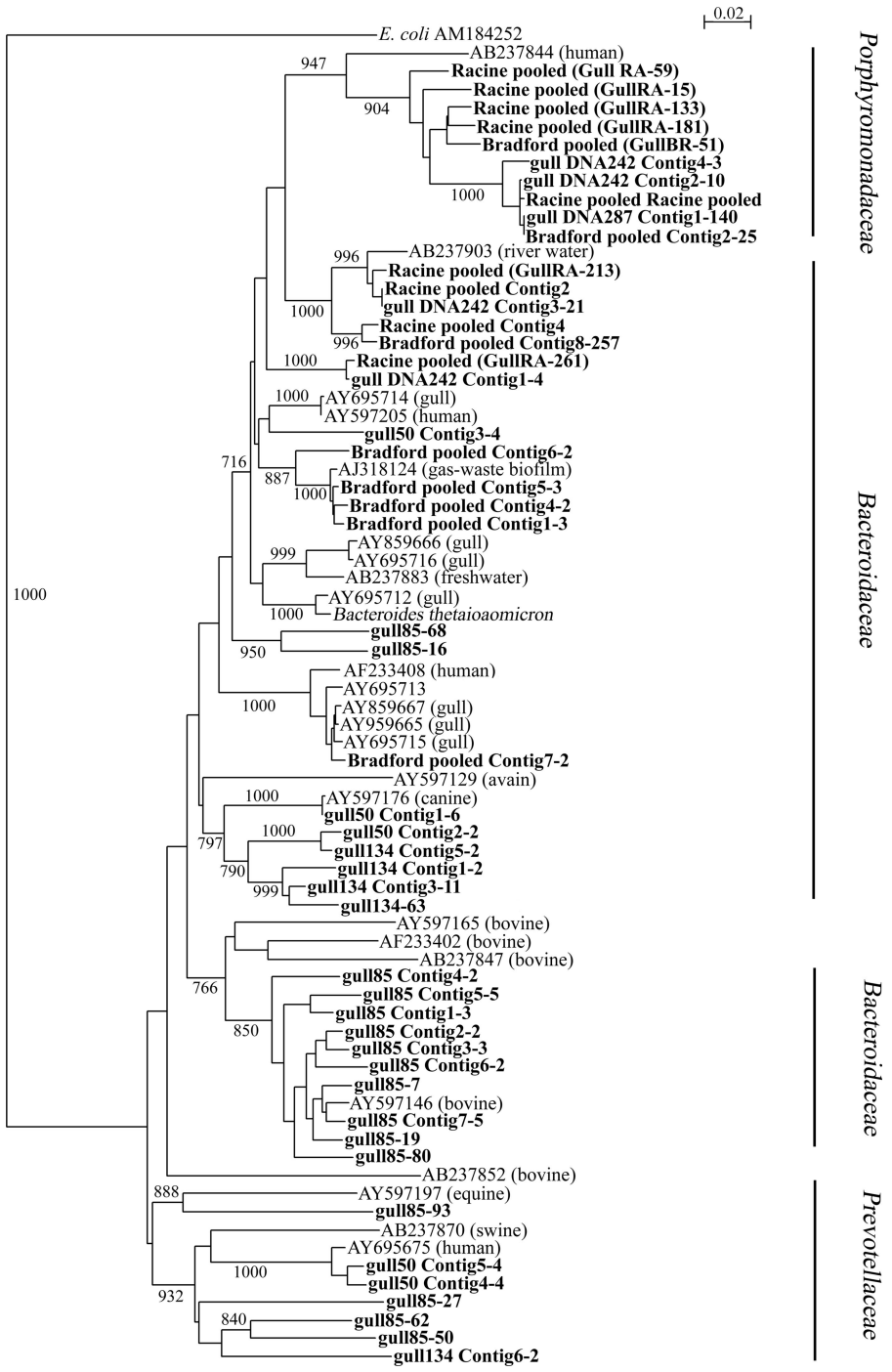

FIG. 1. Neighbor-joining tree for *Bacteroidales* from gulls. Designations for consensus sequences of OTUs at the 97% similarity level contain "Contig," and the number of sequences represented in each OTU is indicated after the hyphen in the designation. Bootstrap values for major branches greater than 700 are shown.

Bacteroides spp. in the clone libraries varied slightly, with the gull libraries having the lowest percentage. Overall, in the gull libraries 43.5% of the clones were identified as *Bacteroides* spp., whereas in the goose, raccoon, canine, and sewage libraries 75.7%, 82.7%, 90.8%, and 66.5% of the clones, respectively, were identified as *Bacteroides* spp. The percentages of sequences that were identified as sequences of members of *Bacteroidaceae* are shown in Table 2. Not all of these sequences were identified as sequences of *Bacteroides* spp. at the 97% identity level, which was used as the cutoff for inclusion in the dendrogram. An overview dendrogram of *Bacteroides* spp. from these sources is shown in Fig. 3. The sequences from gulls collected at northern Lake Michigan beaches clustered more closely with each other than with sequences from other

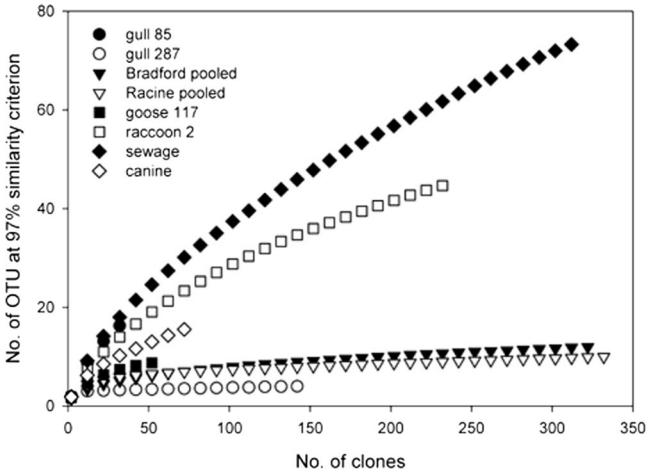

FIG. 2. Rarefaction analysis of clone libraries from different sources. Data for the most diverse library from each individual animal and the two pooled gull libraries are shown. The sewage and gull 85 curves overlap. The OTU criterion used was 97% similarity.

sources, whereas the sequences from gulls collected at the southern beaches were intermixed with sequences from canine, raccoon, and sewage sources in some cases. In addition, there were some clades that were dominated by one source and had a low number of sequences from other sources. Bootstrap values did not support the majority of branches, except for small groups at the termini of larger branches; the members of these small groups were often from one host source but were not necessarily from the same animal.

A phylogenetic tree with members of the *Porphyromonadaceae* was also constructed to examine the relationship between the sequences recovered from gulls and the sequences recovered from other sources. Not all clone libraries were found to have members belonging to this family. The canine library, three goose libraries, and one raccoon library lacked sequences identified as *Porphyromonadaceae* sequences. Gull sequences were segregated together in a closely related clade, and sewage and raccoon sequences also clustered together (Fig. 4). The sequence of a previously characterized *Parabacteroides* sp. from a human source (accession no. AB237844) was more closely related to other previously characterized sequences and one consensus sequence from sewage than to the gull sequences.

Detection of human-specific *Bacteroides* spp. using HF183F in gull samples. To determine if gulls carried *Bacteroides* spp. that could amplify with the HF183F primer, PCR was carried out with all gull samples from the southern Lake Michigan beaches and selected samples (samples in which *Bacteroidales* were detected using the Bac32F and Bac708R primers) from northern Lake Michigan beaches. All 424 gull samples that were tested were negative for human-specific *Bacteroides* spp. In addition to PCR screening, BLAST searches for the HF183F primer sequence were also carried out for 1,747 clones from 14 different libraries. There were no matches in the gull libraries. Three separate goose libraries, a canine library, and the two raccoon libraries were also negative for sequences matching the HF183F primer. Interestingly, the

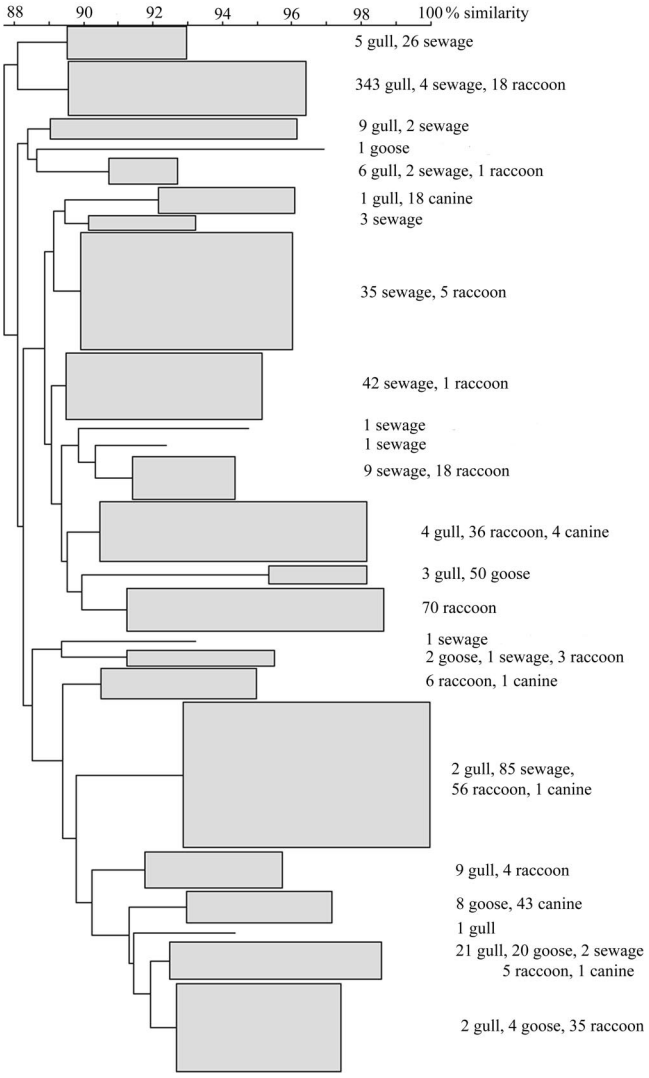

FIG. 3. Dendrogram for cloned sequences identified as *Bacteroides* spp. from gull (n = 406), goose (n = 85), raccoon (n = 258), canine (n = 69), and sewage (n = 214) sources. The boxes represent collapsed branches. The number of sequences from each source is shown to the right of each box.

sewage library was found to have only 26 of 317 sequences that perfectly matched the HF183F primer.

DISCUSSION

Gulls impact beach water quality by introducing fecal indicator organisms and, potentially, pathogens into recreational waters. In particular, human pathogens, such as *Campylobacter*, *Salmonella*, and *Listeria*, have been isolated from ring-billed gull (*Laurus delawarensis*) fecal material (16, 27). Further, high levels of fecal indicator organisms deposited locally (e.g., where water quality samples are collected) by gulls may mask contamination from more serious regional sources, such as sewage overflows released into estuaries and rivers (24). Evidence of whether large numbers of gulls roosting on Lake Michigan and other beaches are responsible for contamination

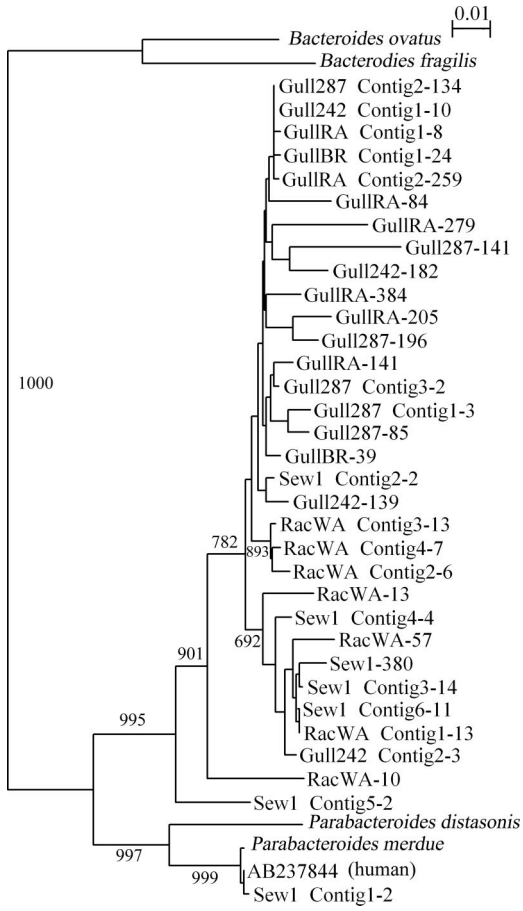

FIG. 4. Neighbor-joining tree for *Porphyromonadaceae* from gull, raccoon, and sewage sources. Designations for consensus sequences of OTUs at the 98% similarity level contain "Contig," and the number of sequences represented in each OTU is indicated after the hyphen in the designation. Bootstrap values for major branches greater than 700 are shown.

of recreational waters would be useful for beach managers who are charged with mitigating sources of pollution and protecting public health.

Members of the order *Bacteroidales* are potentially useful indicators of fecal contamination because they generally are found in high numbers in fecal material of warm-blooded animals and are unlikely to survive in the beach environment (3, 17). Microbial source tracking using *Bacteroides* spp. was attempted by Kreader (17) and later by Bernhard and Field (3), who identified unique sequences in the *Bacteroides* 16S rRNA gene from human and bovine waste. Sequencing of clone libraries revealed that sequences of members of the broader *Bacteroidales* group, rather than exclusively *Bacteroides* spp., are amplified with primers originally targeting total *Bacteroides* spp. (3, 9).

We found that not all gulls carried *Bacteroidales*. There was a low occurrence of these bacteria overall, but they were found in at least some gulls at each beach tested. The percentage of samples from geese that were positive was also low, suggesting that *Bacteroidales* are not common in the avian population. Previous reports have also noted the absence of *Bacteroidales* in gulls and other avian sources (6, 12). It did not appear that the age of a fecal sample or the presence of inhibitors was responsible for the low occurrence in this study, since the southern Lake Michigan samples all contained culturable *E. coli* (data not shown) and were positive for *E. coli* as determined by PCR, which was used as a control. Samples that were negative for *E. coli* were not included in this study; the only exceptions were three samples from northern Lake Michigan beaches that were positive for *Bacteroidales* but negative for *E. coli*. We found that the percentage of *Bacteroidales*-positive fecal samples from gulls at northern Lake Michigan beaches was higher than what was found at southern Lake Michigan beaches. This could be due to the fact that fewer samples were taken at the northern beaches than at the southern beaches or might be because gulls in the northern Lake Michigan county consume different diets than gulls in the southern Lake Michigan counties.

In gull samples, we found members of three of the four families in the order *Bacteroidales*: *Bacteroidaceae*, *Prevotellaceae*, and *Porphyromonadaceae*. Previous studies using the same primers (3) have demonstrated the presence of mainly *Bacteroides* and *Prevotella* spp. (9, 19, 25). We found that *Parabacteroides* sequences were also amplified readily from gulls. *Parabacteroides* spp. were found in two pooled ($n = 5$ and $n = 6$) and two individual samples from southern Lake Michigan gulls, but not in northern Lake Michigan gulls. Only three individual fecal samples from northern Lake Michigan gulls were used to construct libraries; therefore, the presence of *Parabacteroides* spp. in gulls at northern beaches cannot be ruled out without more extensive sampling. In other published reports (3, 9, 18, 19, 25) that generated *Bacteroidales* 16S rRNA gene libraries from sources of fecal pollution, only 17 of 386 submitted sequences showed >80% identity to the family *Porphyromonadaceae*, only 5 of these sequences were identified as *Parabacteroides* sequences, and only one sequence had 100% identity to a *Parabacteroides* sequence (GenBank accession number AB237844, isolated from human feces). We found that the gull *Parabacteroides* sequences clustered together and appeared to be relatively distinct from sequences recovered from sewage and raccoons (Fig. 4). *Parabacteroides* may be unique to certain hosts and warrant further investigation in the gull population.

The *Bacteroidales* is a very deep order and is underexplored in terms of diversity (9, 18). We characterized *Bacteroidales* from the fecal material of a single gull and from pooled gull fecal samples and found similar levels of diversity in both types of samples (Table 3). This demonstrates that there is a high level of diversity in the *Bacteroidales* in a single gull, which should be considered when studies to characterize fecal bacterial populations are designed. The sequencing effort for most gull libraries appeared to capture the majority of *Bacteroidales* within each library, with deeper sequencing showing more microdiversity but a limited number of new OTUs at a similarity level of 97% or greater (Fig. 2). Most gull sequences from northern Lake Michigan and the inland lake (northeastern Wisconsin) clustered together, and sequences from gulls at southern Wisconsin beaches clustered with each other, suggesting that there may be some geographic effects on population structure. The overall topography of the gull sequence dendrogram (Fig. 1) suggests that southern and northern Wis-

consin gulls may be separate reservoirs. Further, the pattern on the dendrogram demonstrated that the Lake Michigan gull sequences were dispersed among a wide range of previously characterized sequences from gulls and various hosts, including humans, cows, chickens, horses, and swine, from other studies. It may be that geographic proximity plays a larger role than the host gut environment (including diet) in the *Bacteroidales* population structure in gulls. Alternatively, these findings may be a result of underrepresentation of gulls in general or may have been influenced by the sampling strategy. It may be that sampling more beaches or sampling in different years would yield additional highly similar clusters that appear to be distinct from clusters characterized previously.

We compared the gull sample sequences to the sequences from raccoons, geese, canines, and human sewage. Rarefaction analysis suggests that the *Bacteroidales* in the other sources are more diverse than the *Bacteroidales* in gulls. Interestingly, these sources have been shown to consistently carry *Bacteroidales*, whereas the gull and goose sources were found to have a low overall occurrence of *Bacteroidales* (6, 12). There was a large amount of microdiversity within a single animal, which was represented by small clusters with high bootstrap values (data not shown). Overall, the host groups characterized in this study were dispersed across the dendrogram, and some clades were dominated (but not exclusively) by one host (Fig. 3). Fogerty and Voytak (12) used terminal restriction fragment length polymorphism analysis and found that most of their data were grouped by source, suggesting that there is potential for using *Bacteroides* spp. as a source tracking tool (5). Dick et al. (9) reported both endemic and cosmopolitan distributions of *Bacteriodales* bacteria. Likewise, Lamendella et al. (18) reported that *Bacteriodetes* from multiple sources clustered closely together, further illustrating the cosmopolitan nature of the distribution. Further study is necessary to characterize the global distribution of *Bacteroidales* and determine if small changes in sequences define distinct populations.

Previous studies have found that gull *Bacteroidales* sequences fall in the same cluster as human sequences (7), suggesting that gulls may carry strains that cross-react with human-specific *Bacteroides* markers, which are used extensively for source tracking. We found no cross-reaction with the HF183F primer in 424 gull samples from five different beaches. These findings are important since gulls can confound beach water quality testing by contributing fecal indicator bacteria, and it is important to distinguish gull sources from human sources, which are known to be a serious health risk.

Bacteroidales DNA was detected in all eight canine samples. Further studies with a larger sample should be conducted to determine if this finding can be generalized to all canine fecal waste. We found that the canine *Bacteroidales* sequences clustered more closely with each other than with other sequences, whereas raccoon and sewage sequences were more commonly intermixed in small closely related clades. These results may reflect geographic differences since the canine sample was from a northern Wisconsin county and the sewage and raccoon samples were from a southern Wisconsin county. Alternatively, these findings might suggest that host factors influence population structure. Dick et al. (9) suggested that animals with similar diets may carry *Bacteroidales* that are closely related. Further characterization of the *Bacteroidales* population within canine hosts is necessary to make these determinations.

The relatively low percentages of avian samples from which *Bacteroidales* could be amplified (e.g., 12.6% of gull samples and 16.6% of goose samples) do not rule out the possibility that members of this order could be used as a bird-specific marker. We identified *Parabacteroides* spp. that were not present in other sources in this study and have not been found in other studies of *Bacteriodales* populations. Alternatively, the absence or low occurrence of *Bacteroidales* in gulls might support the use of *Bacteriodales* as an alternative indicator for fecal pollution at beaches. Gulls have been found to carry high levels of *E. coli* (11), which can confound water testing due to local fecal pollution inputs. In addition, recent reports have suggested that other bacteria may be more suitable for specifically identifying gull fecal material (22). Multiple markers might be the most reliable tools for monitoring and source tracking. Further studies with a larger sample base are needed to fully elucidate the population structure of *Bacteroidales* in gulls in relation to other hosts to advance microbial source tracking efforts at recreational beaches.

ACKNOWLEDGMENTS

We thank Caitlin Scopel and Ola Olapade for assistance with field and laboratory work.

This work was funded by the University of Wisconsin Sea Grant Institute under a grant from the National Sea Grant College Program, National Oceanic and Atmospheric Administration, U.S. Department of Commerce (grant NA06OAR4170011, project R/UC-2). This work was also funded by the National Science Foundation REU Program (REU site DBI 0753525) and the University of Wisconsin—Oshkosh Faculty Development Collaborative Grant Program.

REFERENCES

1. **Ahmed, W., J. Stewart, D. Powell, and T. Gardner.** 2008. Evaluation of *Bacteroides* markers for the detection of human faecal pollution. Lett. Appl. Microbiol. **46:**237–242.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Bernhard, A. E., and K. G. Field.** 2000. Identification of nonpoint sources of fecal pollution in coastal waters by using host-specific 16S ribosomal DNA genetic markers from fecal anaerobes. Appl. Environ. Microbiol. **66:**1587–1594.
4. **Bernhard, A. E., and K. G. Field.** 2000. A PCR assay to discriminate human and ruminant feces on the basis of host differences in *Bacteroides-Prevotella* genes encoding 16S rRNA. Appl. Environ. Microbiol. **66:**4571–4574.
5. **Bower, P. A., C. O. Scopel, E. T. Jensen, M. M. Depas, and S. L. McLellan.** 2005. Detection of genetic markers of fecal indicator bacteria in Lake Michigan and determination of their relationship to *Escherichia coli* densities using standard microbiological methods. Appl. Environ. Microbiol. **71:**8305–8313.
6. **Carson, C. A., J. M. Christiansen, H. Yampara-Iquise, V. W. Benson, C. Baffaut, J. V. Davis, R. R. Broz, W. B. Kurtz, W. M. Rogers, and W. H. Fales.** 2005. Specificity of a *Bacteroides thetaiotaomicron* marker for human feces. Appl. Environ. Microbiol. **71:**4945–4949.
7. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, S. A. Kulam, D. M. McGarrell, G. M. Garrity, and J. M. Tiedje.** 2005. The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res. **33:**D294–D296.
8. **Cole, J. R., B. Chai, T. L. Marsh, R. J. Farris, Q. Wang, S. A. Kulam, S. Chandra, D. M. McGarrell, T. M. Schmidt, G. M. Garrity, and J. M. Tiedje.** 2003. The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res. **31:**442–443.
9. **Dick, L. K., A. E. Bernhard, T. J. Brodeur, J. W. Santo Domingo, J. M. Simpson, S. P. Walters, and K. G. Field.** 2005. Host distributions of uncultivated fecal *Bacteroidales* bacteria reveal genetic markers for fecal source identification. Appl. Environ. Microbiol. **71:**3184–3191.
10. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8:**186–194.

11. **Fogarty, L. R., S. K. Haack, M. J. Wolcott, and R. L. Whitman.** 2003. Abundance and characteristics of the recreational water quality indicator bacteria *Escherichia coli* and enterococci in gull faeces. J. Appl. Microbiol. **94:**865–878.
12. **Fogarty, L. R., and M. A. Voytek.** 2005. Comparison of *Bacteroides-Prevotella* 16S rRNA genetic markers for fecal samples from different animal species. Appl. Environ. Microbiol. **71:**5999–6007.
13. **Haack, S. K., L. R. Fogarty, and C. Wright.** 2003. *Escherichia coli* and enterococci at beaches in the Grand Traverse Bay, Lake Michigan: sources, characteristics, and environmental pathways. Environ. Sci. Technol. **37:**3275–3282.
14. **Huang, X. Q., and A. Madan.** 1999. CAP3: a DNA sequence assembly program. Genome Res. **9:**868–877.
15. **Kildare, B. J., C. M. Leutenegger, B. S. McSwain, D. G. Bambic, V. B. Rajal, and S. Wuertz.** 2007. 16S rRNA-based assays for quantitative detection of universal, human-, cow-, and dog-specific fecal *Bacteroidales*: a Bayesian approach. Water Res. **41:**3701–3715.
16. **Kinzelman, J., S. McLellan, A. Amick, J. Preedit, C. Scopel, O. Olapade, S. Gradus, A. Singh, and G. Sedmak.** 2008. Identification of human enteric pathogens in gull feces at southwestern Lake Michigan bathing beaches. Can. J. Microbiol. **54:**1006–1015.
17. **Kreader, C. A.** 1995. Design and evaluation of *Bacteroides* DNA probes for the specific detection of human fecal pollution. Appl. Environ. Microbiol. **61:**1171–1179.
18. **Lamendella, R., J. W. Domingo, D. B. Oerther, J. R. Vogel, and D. M. Stoeckel.** 2007. Assessment of fecal pollution sources in a small northern-plains watershed using PCR and phylogenetic analyses of *Bacteroidetes* 16S rRNA gene. FEMS Microbiol. Ecol. **59:**651–660.
19. **Layton, A., L. McKay, D. Williams, V. Garrett, R. Gentry, and G. Sayler.** 2006. Development of *Bacteroides* 16S rRNA gene TaqMan-based real-time PCR assays for estimation of total, human, and bovine fecal pollution in water. Appl. Environ. Microbiol. **72:**4214–4224.
20. **Levesque, B., P. Brousseau, F. Bernier, E. Dewailly, and J. Joly.** 2000. Study of the bacterial content of ring-billed gull droppings in relation to recreational water quality. Water Res. **34:**1089–1096.
21. **Lévesque, B., P. Brousseau, P. Simard, E. Dewailly, M. Meisels, D. Ramsay, and J. Joly.** 1993. Impact of the ring-billed gull (*Larus delawarensis*) on the microbiological quality of recreational water. Appl. Environ. Microbiol. **59:**1228–1230.
22. **Lu, J., J. W. Santo Domingo, R. Lamendella, T. Edge, and S. Hill.** 2008. Phylogenetic diversity and molecular detection of bacteria in gull feces. Appl. Environ. Microbiol. **74:**3969–3976.
23. **Matsuki, T., K. Watanabe, J. Fujimoto, Y. Miyamoto, T. Takada, K. Matsumoto, H. Oyaizu, and R. Tanaka.** 2002. Development of 16S rRNA-gene-targeted group-specific primers for the detection and identification of predominant bacteria in human feces. Appl. Environ. Microbiol. **68:**5445–5451.
24. **McLellan, S. L., and A. K. Salmore.** 2003. Evidence for localized bacterial loading as the cause of chronic beach closings in a freshwater marina. Water Res. **37:**2700–2708.
25. **Okabe, S., and Y. Shimazu.** 2007. Persistence of host-specific *Bacteroides-Prevotella* 16S rRNA genetic markers in environmental waters: effects of temperature and salinity. Appl. Microbiol. Biotechnol. **76:**935–944.
26. **Perriere, G., and M. Gouy.** 1996. WWW-query: an on-line retrieval system for biological sequence banks. Biochimie **78:**364–369.
27. **Quessy, S., and S. Messier.** 1992. Prevalence of *Salmonella* spp., *Campylobacter* spp. and *Listeria* spp. in ring-billed gulls (*Larus delawarensis*). J. Wildl. Dis. **28:**526–531.
28. **Sampson, R. W., S. A. Swiatnicki, C. M. McDermott, and G. T. Kleinheinz.** 2005. *E. coli* at Lake Superior recreational beaches. J. Great Lakes Res. **31:**116–121.
29. **Santoro, A. E., and A. B. Boehm.** 2007. Frequent occurrence of the human-specific *Bacteroides* fecal marker at an open coast marine beach: relationship to waves, tides and traditional indicators. Environ. Microbiol. **9:**2038–2049.
30. **Savichtcheva, O., N. Okayama, and S. Okabe.** 2007. Relationships between *Bacteroides* 16S rRNA genetic markers and presence of bacterial enteric pathogens and conventional fecal indicators. Water Res. **41:**3615–3628.
31. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71:**1501–1506.
32. **Thompson, J. D., D. G. Higgins, and T. J. Gibson.** 1994. Clustal-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22:**4673–4680.
33. **U.S. Environmental Protection Agency.** 2003. 2003-2008 EPA strategic plan: direction for the future. U.S. EPA Office of Water, Washington, DC.
34. **U.S. Environmental Protection Agency.** 1986. Ambient water quality criteria for bacteria. EPA 440/5-84-002. Office of Water Regulation and Standards, Washington, DC.
35. **Wade, T. J., R. L. Calderon, K. P. Brenner, E. Sams, M. Beach, R. Haugland, L. Wymer, and A. P. Dufour.** 2008. High sensitivity of children to swimming-associated gastrointestinal illness: results using a rapid assay of recreational water quality. Epidemiology **19:**375–383.
36. **Wade, T. J., R. L. Calderon, E. Sams, M. Beach, K. P. Brenner, A. H. Williams, and A. P. Dufour.** 2006. Rapidly measured indicators of recreational water quality are predictive of swimming-associated gastrointestinal illness. Environ. Health Perspect. **114:**24–28.
37. **Wang, Q., G. M. Garrity, J. M. Tiedje, and J. R. Cole.** 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73:**5261–5267.